ORIGINAL ARTICLE

Diversity of viruses in *Ixodes ricinus*, and characterization of a neurotropic strain of Eyach virus

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Abstract

Ticks transmit more pathogens—including bacteria, parasites and viruses—than any other arthropod vector. Although the epidemiological status of many tick-borne bacteria is very well characterized, tick-borne viruses are still relatively under-studied. Recently, several novel tick-borne viruses have been isolated from human febrile illnesses following tick bites, indicating the existence of other potential new and unknown tick-borne viruses. We used high-throughput sequencing to analyse the virome of *Ixodes ricinus*, the main vector of tick-borne pathogens in Europe. The majority of collected viral sequences were assigned to two potentially novel *Nairovirus* and *Phlebovirus* viruses, with prevalence rates ranging from 3.95% to 23.88% in adults and estimated to be between 0.14% and 72.16% in nymphs. These viruses could not be isolated from the brains of inoculated immunocompromised mice, perhaps indicating that they are unable to infect vertebrates. Within the *I. ricinus* virome, we also identified contigs with >90% identity to the known Eyach virus. Initially isolated in the 1980s, this virus was indirectly associated with human disease, but had never been extensively studied. Eyach virus prevalence varied between 0.07% and 5.26% in ticks from the French Ardennes and Alsace regions. Eyach virus was successfully isolated following intracerebral inoculation of immunocompromised mice with Eyach virus-positive tick extracts. This virus was also able to multiply and persist in the blood of immunocompetent mice inoculated by intraperitoneal injection, and caused brain infections in three of nine juveniles, without any obvious deleterious effects.

Keywords: Bunyaviridae, Eyach virus, Flaviviridae, *Ixodes ricinus*, metagenomics, Next Generation Sequencing, Reoviridae, Rhabdoviridae, tick, virus

Original Submission: 8 January 2016; **Revised Submission:** 26 February 2016; **Accepted:** 26 February 2016

Article published online: 5 March 2016

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Importance

Due to environmental changes, the global incidence of tick-borne disease is increasing worldwide, thus tick-borne diseases have been listed alongside diseases with high emergence risk. Several tick-borne pathogens have already emerged in certain geographical regions, such as tick-borne encephalitis virus and Crimean–Congo haemorrhagic fever virus, and novel tick-borne pathogens are continually being discovered. A proportion of these viruses are zoonotic (severe fever with thrombocytopenia syndrome virus in China, the Heartland virus and the Bourbon virus in the USA) but the pathogenicity of many others is poorly documented. Consequently, it is extremely important to determine the pathogenicity of these viruses, and their potential involvement in undiagnosed animal or human febrile illness or encephalitis.

http://dx.doi.org/10.1016/j.nmni.2016.02.012

Introduction

Ticks are widespread throughout Europe and are the primary arthropod vector of both human and domestic animal disease agents [1]. In terms of public health, the most important European tick is *Ixodes ricinus*, the vector of the Lyme borreliosis bacteria [2,3]. *Ixodes ricinus* can transmit many varieties of pathogens, including bacteria, parasites and viruses, due to specific biological adaptations and its capacity to feed on numerous different animal species. The most prevalent tick-borne disease transmitted by *I. ricinus* is Lyme borreliosis, with over 85 000 new European cases annually [2,3]. However, patients bitten by ticks can also be infected by many other zoonotic pathogens, including parasites, viruses and other bacteria [4,5]. Some of these pathogens were initially identified in ticks decades before their association with human disease (such as *Borrelia miyamotoi*), whereas others have only been discovered very recently (such as the Bourbon virus). The global incidence of tick-borne disease is increasing worldwide as the result of environmental changes, so tick-borne diseases are now highlighted as having significant emergence risk [6–9]. Several tick-borne pathogens have already emerged in specific geographical regions, such as the tick-borne encephalitis virus (TBEV), louping ill virus, Powassan virus, deer tick virus, severe fever with thrombocytopenia syndrome virus and Crimean–Congo haemorrhagic fever virus [10–14], whereas novel tick-borne pathogens are continually being discovered [15–18]. These factors highlight the importance of studying viral epidemiology in tick populations.

Although ticks have the potential to transmit many different viruses, most studies surveying tick-borne pathogens in Europe have focused on bacterial and/or parasitic pathogens. Numerous reports detailing parasitic or bacterial prevalence in either European ticks or animal reservoirs are published every year [19–23]. For viruses however, the situation is completely different. For instance, even though several tick-borne encephalitis cases have been reported in France [24,25], recent data on TBEV prevalence in *I. ricinus* or animal reservoirs in France do not exist. Moreover, no data are available regarding the prevalence of other tick-borne viruses in Europe. This lack of knowledge and relevant data within ticks is surprising, but may be explained by the fact that it is far easier to detect bacterial and parasitic DNA, because they possess the conserved rRNA genes that are most often targeted by broad-range molecular testing. Subsequently, many laboratories, including ours, have focused their research on bacteria and parasites rather than viruses.

In this study, we aimed to describe the global picture of viruses carried by French *I. ricinus*, using RNA deep sequencing to identify and better characterize both DNA and RNA viruses that replicate in ticks. We identified 545 assembled contigs related to eukaryotic viruses. The vast majority of hits mapped to families known to include arboviruses, and the greatest number of contigs probably designated a novel *Nairovirus* and a new *Phlebovirus*. The only known virus identified in the ticks was the Eyach virus, first isolated in Europe in the 1980s, and which has since been forgotten. Here, we demonstrated its capacity to multiply and persist in the blood of OF1 mice, and its ability to colonize murine brains.

Methods

Tick collection and extract preparation

Questing *I. ricinus* female ticks (268 in Ardennes), male ticks (228 in Ardennes) and nymphs (285 and 1455 in Ardennes and Alsace, respectively) were collected by flagging from northeastern France (Ardennes in 2012 49.574177, 4.802835; Alsace in 2010 47.918066, 7.146111). All collected ticks were washed as previously described [26], nymphs were pooled into groups of 15 individuals (116 pools in total) and adults were individually treated. Before RNA extraction, tick samples were crushed in 300 µL Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. A pathogen-free colony was obtained as follows; females from the field were engorged on rabbits and allowed to lay eggs. DNA and RNA samples were extracted from female ticks and PCRs were performed to test for the presence of *Borrelia* spp., *Bartonella* spp., *Anaplasma* spp., *Rickettsia* spp., *Francisella* spp. and *Coxiella* spp. Only larvae from 'pathogen-free' female ticks were conserved and maintained in our colony before use in high throughput sequencing (HTS) experiments.

Animal and ethical issues

Newborn (72 h old) type I interferon receptor knock-out mice ($IFNAR^{-/-}$, genetic background: A129SvEvBrd) [27]) kindly provided by Dr Damien Vitour (Virology Unit, Animal Health Laboratory, ANSES) were used for viral isolation from tick extracts. Females (4–5 weeks old) and newborn OF1 mice (72 h old) (Charles River Company, Wilmington, MA, USA) were used for studying the course of the Eyach virus infection. Animal experiments were carried out in strict accordance with appropriate animal care practices as recommended by European guidelines. Protocols were approved by the ANSES-ENVA-UPEC Ethics Committee for Animal Experimentation (Agreement Number: 13-021).

RNA extraction

Crushed ticks (individual adults or pooled nymphs) were divided into two equal samples. One half was directly frozen

at −80°C for subsequent viral isolation, the other half was used for total RNA extraction using the Nucleospin RNA II kit (Macherey-Nagel, Duren, Germany) following the manufacturer's instructions.

High throughput sequencing and bioinformatic analysis of sequences obtained from tick extracts

RNA samples were pooled relating to their geographical area (one Ardennes pool and one Alsace pool). The pooled RNA samples were retro-transcribed to cDNA, and randomly amplified using the multiple displacement amplification (MDA) protocol with phi29 polymerase and random hexamers as described previously [26]. Library preparations and sequencing with an Illumina HiSeq2000 sequencer were outsourced to DNAVision (Charleroi, Belgium). Wild and pathogen-free samples were sequenced to a depth of 200×10^6 (Alsace sample), 131×10^6 (Ardennes sample) and 62×10^6 (pathogen-free sample) paired-end reads of 101 bp. Raw sequence reads were trimmed according to their quality score. At the time of analysis, there was no publicly available reference genome for *I. ricinus*, so tick sequences were removed from the analysis by subtracting sequences identical to those derived from a pathogen-free reference sample using the SOAP2 aligner tool [28]. Finally, *de novo* assembly was performed on all remaining reads (5.2×10^6 and 7.8×10^6 for the Ardennes and Alsace tick samples, respectively) using CLC AssemblyCell software (version 4.0), producing 16 094 and 174 841 contigs in the Ardennes and Alsace tick samples, respectively. Taxonomic assignation of these contigs was achieved by successive sequence alignment using nucleotide and protein databases and the BLAST algorithm. Contigs were assigned the closest known taxonomy according to their identity percentage, and distant alignments were not considered.

Virus prevalence identification in ticks

For all viruses, primers and probes were derived from high throughput contigs (HTS-contigs). Eyach virus RNA was detected in ticks by quantitative RT-PCR targeted to the *vp2* gene (Primer F: 5′-TGGCTGACAACATGACGGATA-3′; Primer R: 5′-GGCCTCACGATACTTTCGATT-3′); probe (5′-FAM-ACGGGCTCGGTACTTCGGTTGAGAT-BHQ1-3′).

The quantitative RT-PCRs were performed in a final volume of 20 µL using Lightcycler 480 RNA Master Hydrolysis Probes (Roche Applied Science, Penzberg, Germany), with primers at 0.5 µM, the probe at 0.25 µM and 2 µL of RNA (quantity range from 200 to 400 ng). Thermal cycling conditions were 63°C for 3 min, 95°C for 30 sec, 45 cycles at 95°C for 10 sec, and 60°C for 30 sec and one final cooling cycle at 40°C for 30 sec. Each RNA sample was run in duplicate.

To detect the novel *Nairovirus* and *Phlebovirus* RNA, the cDNA synthesis step was performed with random hexamers (Superscript III RT; Invitrogen Inc., Carlsbad, CA, USA). Amplification of L, M and S segments from each new *Nairovirus* and *Phlebovirus* was performed using 22 specific primer pairs (Table 1) designed from HTS-contigs. All PCR amplifications were performed by using the *Taq* Core kit (MPBio, Illkirch, France) following the manufacturer's instructions.

Viral isolation from positive tick extracts

Newborn IFNAR$^{-/-}$ mice (48 to 72 h old) were directly inoculated with 20 µL of tick extracts positive for either the novel *Nairovirus* or Eyach virus (pool of ticks numeros E48 and E134). Mice were observed daily, and if paralysis occurred, mice were euthanized, and their brains were harvested and homogenized in medium. RNA was extracted using the Nucleospin RNA II Macherey-Nagel kit following the manufacturer's instructions, viral RNA was detected by quantitative RT-PCR as described above.

Full genome sequence and phylogenetic analysis of Eyach virus

Purified RNA from mouse brains infected with Eyach-positive tick extracts was amplified as previously described for tick RNA and sequenced on a Life Technologies Proton sequencer using a single chip (around 80×10^6 reads). Genome sequences were derived from *de novo* assembled contigs. For phylogenetic analysis, HTS-contig sequences of Eyach virus strains from the RNA-dependent RNA polymerase gene (segment 1) from French ticks (deposited in GenBank, accession numbers KU133666–KU133676, KU133678, KU133679) were compared to seven available GenBank sequences of Eyach virus using maximum likelihood trees (GenBank reference sequences AF343052, EU789374–EU789377, AF282467 and AF343053). The Colorado tick fever virus was selected as an outgroup (GenBank reference sequence NC_004181).

Infection of OF1 mice with Eyach virus isolated from tick extracts

Eyach virus inoculum obtained from the brain homogenates of infected newborn IFNAR$^{-/-}$ mice (2.4×10^5 copy/µL) was intraperitoneally injected into OF1 mice: five females (100 µL) and nine newborn mice (40 µL). For controls, adult and newborn OF1 mice were respectively intraperitoneally inoculated with 100 µL and 40 µL of 1× PBS. Mice (infected and negative controls) were observed and weighed daily until day 21. Recorded clinical signs included significant weight loss, weakness, ruffled fur, hunched posture, ataxia, tremors and occasionally hind leg paralysis.

Blood samples (infected and negative controls) were collected from newborn (40 µL) and adult (100 µL) mice at

TABLE 1. Primers designed from contigs to detect new *Nairovirus* and *Phlebovirus*

Viral genome targeted	Primer name	Sequence (5′–3′)	PCR product size (bp)
Nairovirus from Alsace ticks	L-F-105533	CAGGATTGTCAGTGGCCTTTC	382
	L-R-105533	CACTGAGGGCATGTTCTGTTC	
	M-F-94623	CTGACGTCGACAAGCCCTTC	319
	M-R-94623	GCAGGTCCATGAGTGCATGAC	
	S-F-86211	CGAGCGTTGCTTTTCTGAGGG	276
	S-R-86211	AAGTTCCCAGTGGCGCACAAG	
Phlebovirus from Alsace ticks	L1-F-43969	CATTCCCCGCCTTCCAAGAG	553
	L1-R-43969	TTGGCCTCCACTCCTAGTGTG	
	L2-F-44658	GAGCAGGCCTATGCTCTACAC	433
	L2-R-44658	TCAGGCCGTAGAACTCTAGCC	
	M-F-171339	ACCGCCAAATGCTCATGCCAG	437
	M-R-171339	CAATTGGCCCAGCACCAGAAG	
	S-F-358	CGCACGTGGTCACTTGTCTTC	472
	S-R-358	GTGGGAAGGCTAGGCTCAAC	
Nairovirus from Ardennes ticks	L-F-287	GCCCGAACAACCTTACATGTC	199
	L-R-287	ACATATGCTCTTCAAACAAGTGGG	
	S-F-6797	TGCACTATGTCTGGATCTGTC	112
	S-R-6797	AAGGGTCAGTGTTGGTATGCC	
Phlebovirus from Ardennes ticks	L-F-542	TTGTCCCACCAGTTGAGAAGG	239
	L-R-542	GAAAGGTACTTTGACCTGGCGT	
	M-F-8702	TCGACGGAGAAACAAAGCAGT	140
	M-R-8702	AATGTCCGAGAACTCCTCATC	

days 7, 14 and 21. Mice were killed at day 21, and brains and spleens were harvested.

RNA preparation from mouse tissues and blood

Brains and spleens were individually crushed in 500 µL Dulbecco's modified Eagle's medium before RNA extraction. RNA was extracted from total blood, brain or spleen homogenates using the Nucleospin RNA II Macherey-Nagel kit following the manufacturer's instructions.

Quantification of Eyach virus in mouse tissues and blood

For each RNA sample (brain, spleen, blood), digital RT-PCR was performed to quantify Eyach *vp2* segments. Digital RT-PCR amplifications were performed on a Fluidigm BioMark System using digital array microfluidic chips (Fluidigm Corporation, San Francisco, CA, USA).

Reactions were performed in 8 µL of reaction mixture containing 0.4 µL of sample loading reagent (BioMark), 1.6 µL of Roche RT Mix (Roche), 0.4 µL of RT superscript III (Invitrogen), 0.16µL of ROX reference dye (BioMark), 0.4 µL of Primer stock (mixture of primers and probe at a final concentration of 18 µM for each primer and 4 µM for probe), and 3.1 µL RNA (quantity range from 620 ng to 1240 ng), or water for negative controls. Half of the 8 µL reaction mix was loaded onto the chip with the Integrated Fluidic Circuit controller, effectively partitioning 0.65 µl (equivalent to 0.25 µL RNA; quantity range to 50 ng to 100 ng) into each of the 770 chambers per panel. The one-step digital RT-PCR programme involved a step of 25°C for 10 min, one step of reverse transcription at 50°C for 50 min, followed by a 5-min denaturation step at 95°C, and lastly 45 cycles of 10 sec at 95°C and 30 sec at 60°C. The Digital PCR Analysis software, part of the BioMark System (Fluidigm Corporation) was used to count the number of positive chambers out of the total number of chambers per panel. Poisson distribution was used to estimate the average number of template copies per chamber in a panel. All samples were characterized by a corresponding absolute quantity per microlitre. No positive chambers were observed in the negative controls.

Statistical analysis

Numbers of viral RNA copies in the blood of females at days 7, 14 and 21 were compared using the online Kruskal–Wallis test (Aus Vet Animal Health Service http://epitools.ausvet.com.au/content.php?page=home). The same statistical analyses were performed for newborn mice. Numbers of viral RNA copies in the blood of females compared with newborn mice at day 7, day 14 and day 21 were done using the online Wilcoxon rank sum test. The differences were considered statistically significant if $p < 0.05$.

Results

Ixodes ricinus viral metatranscriptomic analysis

Metatranscriptome analysis was performed on two pooled *I. ricinus* RNA samples: subsequently termed the 'Alsace sample' and the 'Ardennes sample', and detected pathogens including replicating DNA and RNA viruses, including 174 841 and 16 094 assembled contigs were generated for the Alsace and Ardennes samples respectively. Of these contigs, 395 and 150 had best hits corresponding to eukaryote viruses belonging to viral genera comprising eukaryotic viruses of vertebrates and/or arthropods. We excluded plant or fungi viruses for the sake of clarity. Fig. 1(a,b) illustrates the viral family distribution of read counts used to assemble the 395 and 150 contigs. The vast majority of hits mapped to virus families known to replicate in arthropods. Most belonged to *Bunyaviridae* (*Nairovirus*, *Phlebovirus*, *Orthobunyavirus*), *Reoviridae* (*Coltivirus*), *Rhabdoviridae* (*Vesiculovirus*, *Sigmavirus*), *Picornaviridae*-like (*Iflavirus*, Drosophila virus A) families (see Table 2 for the Alsace sample and Table 3 for the Ardennes sample). Hits with high nucleotide identity (94%–100%) were all assigned to the identical *Coltivirus* virus, i.e. Eyach virus. We identified 19 contigs corresponding to the 12 segments normally present within *Coltivirus* genomes. All other hits were distant from known viruses (50% mean nucleotide identity, range 25%–72%), most belonged to families known to replicate in arthropods. The most frequently occurring

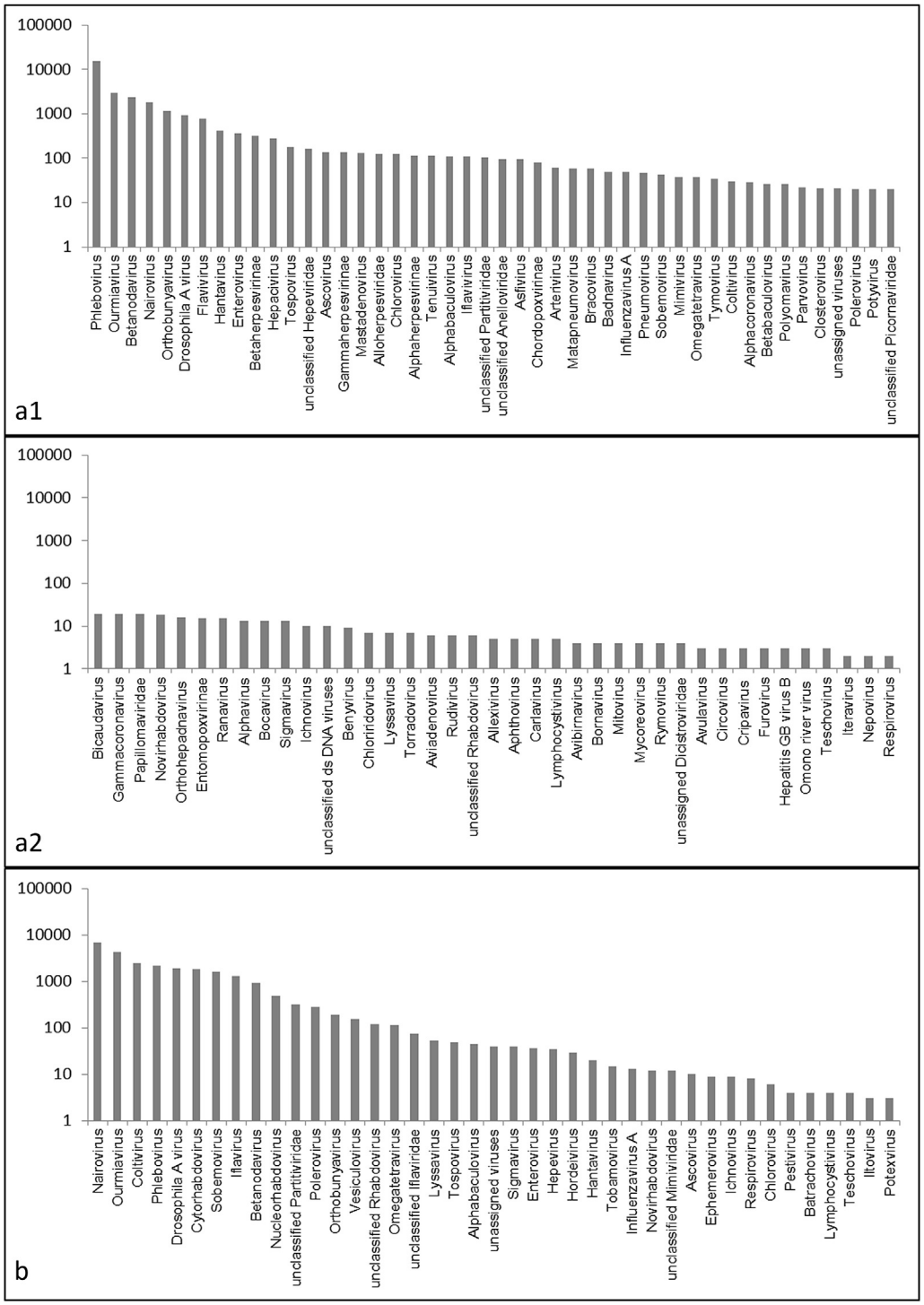

FIG. 1. Number of reads assembled into 395 contigs (Alsace sample) and 150 contigs (Ardennes sample), with best hits corresponding to eukaryotic viruses belonging to genera comprising virus species of vertebrates or arthropods (log scale). (a1 and a2) Alsace ticks; (b) Ardennes ticks.

contigs corresponded to the *Nairovirus* and *Phlebovirus* genera in both the Ardennes and Alsace samples, (68/395 and 39/395 contigs, respectively, for the Alsace sample, and 25/150 and 16/150 contigs, respectively, for the Ardennes sample). These contigs corresponded to all three viral genome segments (L, M and S). None of the contigs corresponded to any of the three viral genome segments from other viral genera belonging to the Bunyaviridae family. We then focused

TABLE 2. Viral contigs detected in *Ixodes ricinus* ticks from Alsace by high-throughput sequencing; best hit result relating to arbovirus family

Family	Genus	Taxonomy (Best Hit Result)	Number of contigs	% homology	Genome (segment or gene)	Contig length (nt)	Number of reads
Reoviridae	Coltivirus	Eyach	5	27.20–60.00	Segment 1 (Polymerase)	114–482	24
		Eyach	1	61.49	Segment 2 (VP2)	211	4
		Eyach	1	45.45	Segment 3 (VP3)	139	2
Bunyaviridae	Hantavirus	Muju or Thottapalayam	2	27.44/30.56	L segment	228/679	230
		Yakeshi or Adler	2	30.23/47.37	S segment	234/530	176
	Nairovirus	Nairobi sheep disease or Crimean–Congo haemorrhagic fever or South Bay or Leopards Hill or Yoque or Kasokero or Keterrah or Dugbe or Hazara or Erve or Thiofara	54	21.98–88.10	L segment	101–1222	1394
		Nairobi sheep disease or Crimean–Congo haemorrhagic fever or Gossas	8	26.62–62.03	M segment	142–1090	284
		Nairobi sheep disease or Erve or Crimean–Congo haemorrhagic fever	6	31.58–43.94	S segment	167–686	158
	Orthobunyavirus	Ilesha or Calovo or Tahyna or Wyeomyia or Iaco or Chatanga or Batai or Ingwavuma	14	24.72–51.16	L segment	133–903	1122
		Guaroa or Restan or Pongola	4	28.68–52.00	M segment	101–521	52
	Phlebovirus	Blacklegged tick or Sandfly fever sicilian or Chagres or Adana or Jacunda or Serra Norte or Rift Valley fever or Odrenisrou or Arumowot or Ixcanal or Aquacate or Karimabad or Chandiru or Uriurana or Razdan or Bhanja	26	25.64–76.52	L segment	101–6189	10891
		Itaituba or Adana or Munguba or Armero or Gissar or Salanga or Rift Valley fever or Chandiru or Arumowot	10	29.91–81.48	M segment	133–1923	4383
		Oriximina or Gabek forest or Toscana	3	39.39–63.59	S segment	176–625	110
	Tospovirus	Calla lily chlorotic spot or Tomato spotted wilt	2	73.68/74.60	L and M segment	486/177	167/8
Rhabdoviridae	Lyssavirus	Rabies or European bat lyssavirus	3	36.84–74.19	gene G, L and N	112–130	7
	Novirhabdovirus	Infectious haematopoietic necrosis or viral haemorrhagic septicaemia virus	3	36.59–50.00	gene G and P	159–262	18
	Sigmavirus	*Drosophila melanogaster* sigma virus	1	72.11	Gene L	533	13
	unclassified	Malpais Spring or Radi	2	41.18/44.44	Gene L and G	124/142	6
Flaviviridae	Flavivirus	Dengue or Mogiana tick or Murray valley encephalitis	6	32.39–60.00	polyprotein	166–419	772
	Hepacivirus	Hepatitis C	4	26.24–64.71	polyprotein	157–585	275
	Hepatitis GB virus B	Hepatitis GB virus B	1	84.09	polyprotein	112	3
Togaviridae	Alphavirus	Western equine encephalitis	3	43.33–80.00	structural protein	170–254	13

on the little-known or new viruses for whom HTS analyses gave the most complete data. Subsequently, we evaluated the prevalence of the Eyach virus, and the likely novel *Nairovirus* and *Phlebovirus* members in ticks, by identifying infected ticks, and then used crushed tick extracts in further isolation and characterization studies.

RNA virus prevalence in *I. ricinus*

The prevalence of the Eyach virus, novel *Nairovirus* and novel *Phlebovirus* RNA viruses was calculated in adult ticks (female and male) from the Ardennes, and was estimated in pooled nymphs from both the Alsace and Ardennes regions.

The prevalence of Eyach virus RNA was 1.87% in female adult ticks (5/268) and 0.88% in adult males (2/228). In the Ardennes samples, 1/19 nymph pools (each comprising 15 nymphs) was positive, indicating that in nymphs, prevalence ranged between 0.35% (if only one nymph was positive per pool) and 5.26% (if all 15 nymphs were positive in each pool). In Alsace ticks, 1/97 nymph pools was positive, with an estimated nymph prevalence ranging between 0.07% and 1.03%.

Concerning the new *Nairovirus*, only the L segment was detected in adult ticks from Ardennes, with a prevalence of 23.88% in females (64/268) and 16.67% in males (38/228). In nymphs from Alsace, all three segments were detected with prevalences ranging between 4.81% and 72.16% (70/97) for the L contig, between 0.14% and 2.06% (2/97) for the M contig, and between 0.48% and 7.22% (7/97) for the S contig. All three segments were present in two nymph pools (E48 and E134). Interestingly, both of these pools were also positive for the Eyach virus. New *Nairovirus* members were not detected in the Ardennes nymphs.

For the new *Phlebovirus* member(s), only the L segment was detected in adult ticks from Ardennes with a prevalence of 5.60% in females (15/268), and 3.95% in males (9/228). In the nymph pools from Ardennes, only the L and M fragments were detected: 13/19 nymph pools were positive for the L segment, indicating that in nymphs, the L-segment prevalence ranged between 4.56% and 68.42%; the M contig was detected in 1/19 nymph pools, with a prevalence between 0.35% and 5.26%. No pools were simultaneously positive for both the L and M fragments. In Alsace nymphs, only the L segment was detected with a prevalence ranging between 2.82% and 42.27% (41/97).

Viral isolation from positive tick extracts

We attempted Eyach virus and new *Nairovirus* isolation from tick extracts positive for all three segments. As no tick extracts carried more than one new *Phlebovirus* segment, we did not

TABLE 3. Viral contigs detected in *Ixodes ricinus* ticks from the Ardennes by high-throughput sequencing; best hit result relating to arbovirus family

Family	Genus	Taxonomy (Best Hit Result)	Number of contigs	% homology	Genome (segment or gene)	Contig length (nt)	Number of reads
Reoviridae	Coltivirus	Eyach	2	95.97/96.94	Segment 1	248/267	12
			4	95.32–99.28	Segment 3	152–361	2277
			1	94.22	Segment 5	1392	77
			3	45.00–96.90	Segment 6	154–1227	55
			3	97.14–100.00	Segment 7	198–328	18
			1	98.93	Segment 8	187	6
			1	97.52	Segment 9	212	5
			1	96.40	Segment 10	276	6
			2	92.57/97.97	Segment 11	202/314	15
			1	95.97	Segment 12	187	3
Bunyaviridae	Hantavirus	Dobrava-Belgrade	1	37.88	M segment	361	20
	Nairovirus	South Bay or Crimean–Congo haemorrhagic fever or Leopards hill or Hazara or Nairobi Sheep Disease	24	22.89–94.59	L segment	102–2699	6704
		Crimean–Congo haemorhagic fever	1	38.60	S segment	173	4
	Orthobunyavirus	Calovo	1	36.67	L segment	197	39
		Zungarococha or Shamonda or Tinaroo	3	26.76–30.88	M segment	232–440	152
	Phlebovirus	Blacklegged tick or Campana or Rift valley fever or Phlebovirus CoAr or Solabo or Solehabad or Uukuniemi	12	38.27–73.85	L segment	141–6159	2167
		Morumbi or Nairobi sheep disease or Chagres or Munguba	4	33.71–67.24	M segment	225–508	37
	Tospovirus	Pepper chlorotic spot	1	47.62	L segment	137	7
		Watermelon silver mottle	1	33.33	M segment	451	30
		Pepper chlorotic spot	1	27.07	S segment	427	12
Rhabdoviridae	Cytorhabdovirus	Barley yellow striate mosaic or Lettuce yellow mottle or Ivy vein banding or Maize yellow striate	4	25.49–62.96	Gene L	127–3300	1838
	Ephemerovirus	Berrimah	1	38.55	Gene G	276	9
	Lyssavirus	Ozernoe or Mokola	2	36.96/67.71	Gene L	125/174	52
	Novirhabdovirus	Infectious haematopoietic necrosis	1	29.21	Gene G	373	12
	Nucleorhabdovirus	Taro vein chlorosis	1	36.03	Gene L	632	205
		Datura yellow vein	1	28.38	Gene G	1023	284
	Sigmavirus	*Drosophila affinis* or *Drosophila ananassae*	2	25.41/67.51	Gene L	405/434	39
	unclassified	Santa Barbara or Morreton or Hybrid snakehead or Siniperca chuatsi or Starry flounder	6	42.86–70.97	Gene L	170–793	95
		Almpiwar	1	40.68	Gene P	397	22
	Vesiculovirus	Chandipura	2	60.84/64.71	Gene L	495/966	56
		Vesicular stomatitis or Maraba	2	31.44/40.70	Gene N	310/838	97
Orthomyxoviridae	Influenzavirus A	Influenza A	1	80.00	Segment 4	119	13
Flaviviridae	Pestivirus	Bovine viral diarrhoea 3	1	96.71	polyprotein	252	4

attempt isolation of this virus. The absence of all three *Phlebovirus* segments in tick extracts may reflect low viral loads, or differences in PCR efficacies.

To isolate the viruses, IFNAR$^{-/-}$ mouse brains were inoculated with tick extracts positive for all three segments of either the new *Nairovirus* or the Eyach virus. All infected mice died 4 days post-infection. Eyach virus was detected by quantitative RT-PCR in RNA extracted from mouse brains infected with Eyach-positive tick extracts, whereas none of the new *Nairovirus* segments were detected by RT-PCR in the brains of mice infected with new *Nairovirus*-positive tick extracts.

Sequence and phylogenetic position of Eyach virus

Sequencing one Eyach virus-positive RNA brain sample generated 17.9 × 10^6 reads with 109 contigs covering the 12 segments of the Eyach viral genome (Table 4). Each Eyach virus consensus segment was submitted to GenBank (Accession numbers: KU163321–KU163332). Contig identities comprised between 86% and 100% compared with the available Eyach virus genome (AF282467.1–AF282478.1). HTS resulted in 93.8% coverage of the entire Eyach genome, with nucleotide identity ranging between 83.3% and 99.2%, depending on the analysed segment (see Supplementary material, Fig. S1).

Phylogenetic analysis of Eyach virus segment 1 contigs (Accession numbers KU133666–KU133676, KU133678, KU133679) revealed that Eyach genotypes identified in the Alsace and Ardennes regions were more closely related to the German Eyach virus isolated from *I. ricinus*, than to other French Eyach viruses isolated from *I. ricinus* and *Ixodes ventalloi* collected in the west of France (Fig. 2).

Eyach viruses exhibit neurotropism in newborn OF1 mice

We then investigated whether the virus could replicate in immunocompetent mice (adults and juveniles) following intraperitoneal inoculation. Infected adults and newborn mice presented no obvious clinical signs of disease (such as weight loss, weakness, ruffled fur, hunched posture, ataxia, tremor) compared with controls.

To assess Eyach viral load in the blood, spleen and brain of each mouse, Eyach virus RNA was evaluated at days 7, 14 and 21 by digital RT-PCR (Fig. 3). Viral Eyach RNA was detected in

TABLE 4. Eyach contigs obtained after intracranial inoculation in mice

Segment Eyach virus (Accession number GenBank)	Number of contigs	% homology	Contig length (nt)	Consensus length (nt)	Segment size (nt)	% segment recovery
1 (KU163321)	12	87–97	105–2470	4280	4349	98
2 (KU163322)	8	94–98	127–2302	3641	3934	93
3 (KU163323)	12	94–98	107–1719	3077	3585	86
4 (KU163324)	14	93–100	102–1358	2937	3156	93
5 (KU163325)	10	86–99	102–2274	2378	2398	99
6 (KU163326)	15	87–99	107–1585	2014	2178	92
7 (KU163327)	4	79–98	102–1752	1841	2139	86
8 (KU163328)	3	96–98	126–1336	1988	2028	98
9 (KU163329)	17	92–99	103–868	1857	1884	99
10 (KU163330)	2	96	128/1970	1858	1879	99
11 (KU163331)	10	88–98	107–914	985	1002	98
12 (KU163332)	2	97/99	170/430	565	678	83

the blood of 4/5 inoculated females. Viral RNA persisted in the blood of the four females until day 21, with no statistical difference in copy numbers between days 7, 14 and 21 (p 0.1637), but was not detected in either the spleen or brain of the five adult females at day 21. Viral RNA was present in the blood of all newborn mice at each time-point, with the highest observed copy number/mL at day 14 (p 0.0344). Viral RNA was present in the spleens of 6/9 newborn mice and in the brains of 3/9 newborn mice at day 21. Eyach viral copies were higher in newborn blood samples than in adult female blood samples (day 7, p 0.042; day 14, p 0.001; day 21, p 0.004).

Discussion

In this study we analysed the virome of *I. ricinus,* the most relevant tick species to animal and human health in Europe. Our ultimate aim was to uncover new or unsuspected tick-borne animal or human viruses in *I. ricinus*. We identified a multitude of diverse eukaryotic viral sequences related to major arbovirus families. Interestingly, most of the eukaryotic viral sequences detected *in I. ricinus* belonged to probable new viruses, as only one previously known virus was identified, the Eyach virus. This had been identified in the 1980s as a potential human pathogen in ticks but has not been studied subsequently. Here, we isolated an Eyach viral strain with neurotropism in mice, confirming that this virus has the capacity to infect vertebrates and traverse the blood–brain barrier. Although we cannot draw conclusions regarding viral pathogenicity from these data, our results lay the foundation for several studies regarding the pathogen itself as well as their possible hosts and reservoirs.

Following the analysis of the two sets of HTS sequences, we obtained contigs belonging to *Bunyaviridae* (*Nairovirus*,

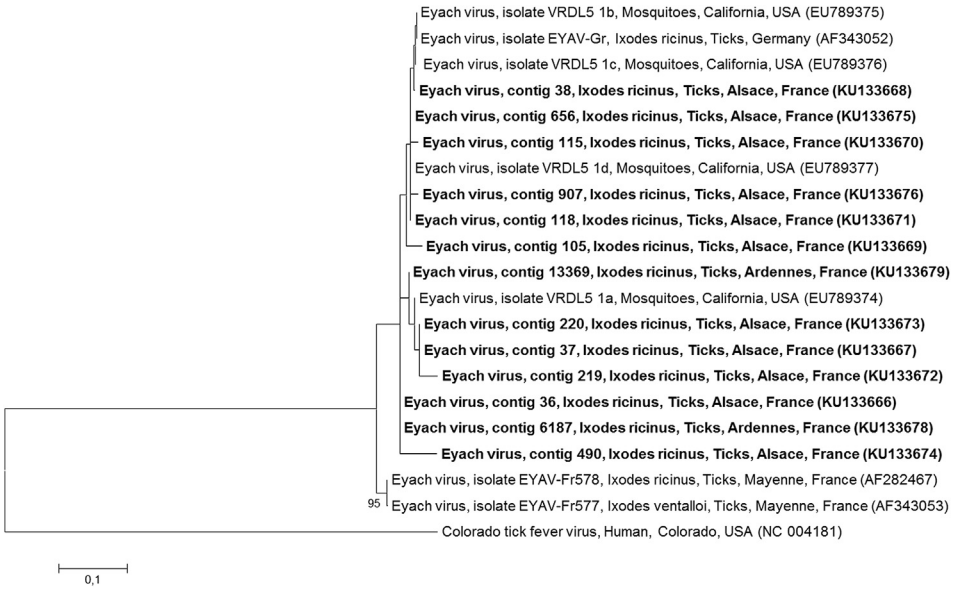

FIG. 2. Maximum likelihood trees of segment 1 of Eyach virus. GenBank accession numbers are given in parentheses. Sequences (contigs) from France are indicated in bold. Numbers represent bootstrap values (%) based on 1000 replications. Trees are rooted with Colorado tick fever virus as an outgroup.

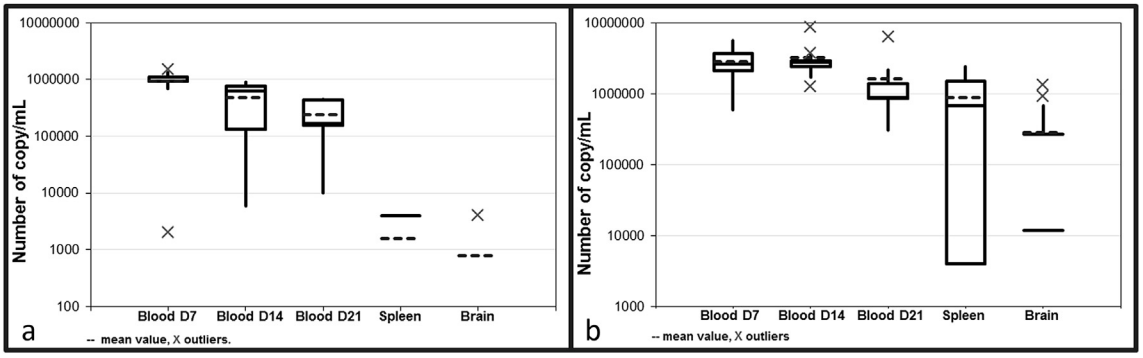

FIG. 3. Box plot representing the range of Eyach viral load as assessed by viral RNA (copy/mL) in the blood at days 7, 14 and 21, in the spleens and brains of five OF1 adult female mice (a) and nine newborn OF1 mice (b).

Phlebovirus, *Orthobunyavirus*), *Reoviridae* (*Coltivirus*), *Rhabdoviridae* (*Vesiculovirus*, *Sigmavirus*) and *Picornaviridae*-like (*Iflavirus*, Drosophila virus A) with nucleotide identity to known viruses ranging from 20% to 100%. Among these contigs, predominant sequences were for viral families, which include arboviruses, suggesting that they are unlikely to represent background noise. Most sequences shared <80% identity with known viruses, potentially indicating new viral species. In both HTS data groups (obtained from Alsace and Ardennes samples), the greatest number of contigs with relatively low percentage identity to known viruses, belonged to viruses from the *Nairovirus* and *Phlebovirus* genera. The *Nairovirus* contigs demonstrated the closest taxonomies to pathogenic tick-transmitted Nairoviruses, such as Nairobi sheep disease virus, Hazara virus, Crimean–Congo haemorrhagic fever virus, or Dugbe virus. Concerning the *Phlebovirus*-related contigs, most were related to non-pathogenic tick-associated viruses, such as Blacklegged tick virus or Uukuniemi virus.

Ticks had relatively high infection rates for both genera in the two geographical areas studied: between 3.95% and 23.88% in adults, and between 0.14% and 72.16% in nymphs. These rates are higher than have typically been reported for other tick-borne viruses, as TBEV prevalence in European ticks is usually between 0.1% and 1.22% [29,30] and Powassan virus prevalence in American ticks is often lower than 2% [31]. However, high *Nairovirus* and *Phlebovirus* tick infection rates have previously been reported (from 5.7% to 23.5%) [14,32,33]. Possible explanations for the high prevalence rates may be either due to extensive bioavailability of susceptible viraemic hosts, or because these viruses can be transovarially transmitted from females to offspring. In the latter case, these viruses could then be considered as endogenous tick viruses, where the ticks themselves play the role of long-term reservoirs [34]. Endogenous tick viruses are not necessarily able to infect vertebrates, and for many, almost nothing is known about their biology or their potential pathogenic effects on humans or animals. The inability to detect sequences corresponding to these new *Nairovirus* after inoculating the brains of immunocompromised mice with positive tick extracts suggests that these viruses are unable to multiply in this particular vertebrate host.

In addition to contigs corresponding to probable viruses, we identified contigs sharing more than 90% identity with another known virus. These contigs were all assigned to the same virus, Eyach virus. This virus, a member of the *Coltivirus* genus within the *Reoviridae* family, was first isolated in *I. ricinus* collected from Germany in 1972 [35]. Subsequently, it was isolated in *I. ventalloi* and *I. ricinus* from France in 1981 [36]. Since this time and despite the lack of surveillance, this virus is evidently still present in France. The Eyach virus was indirectly incriminated in cases of encephalitis and polyradiculoneuritis in the former Czechoslovakia, as antibodies to the virus were identified in sera from patients with a neurological syndrome, but no formal confirmatory viral identification occurred [37]. Nevertheless, solid demonstration of causality is still lacking.

An animal reservoir for the Eyach virus, if it does exist, remains unidentified, even though anti-Eyach virus antibodies have been identified in many animal species in France, including the European rabbit (*Oryctolagus cuniculus*), rodents, sheep, deer and mountain goats [38,39]. We demonstrated that Eyach virus prevalence (0.07%–5.26%) was similar in Ardennes and Alsace ticks and was similar to rates of TBEV, which is endemic in the studied regions [29,30]. So far, no viral RNA has been isolated from animals or humans; however, successful isolation of the virus following intracerebral inoculation of mice with infected tick extract, indicated that the virus could possibly multiply in vertebrate hosts. This result was reinforced by the demonstration that after intraperitoneal inoculation in immunocompetent mice, the virus was able to multiply and persist in the blood for up to 21 days after infection. Finally, we identified the virus in the

brains of suckling mice as evidence of its neurotropism. More advanced analysis of the Eyach virus should be performed to determine the presence of lesions and/or to determine specific cellular tropism in the brain. The long-lasting viraemia of Eyach virus observed in OF1 mice might suggest that rodents could be the natural reservoir of this virus. However, a preliminary prevalence study performed on bank voles collected in the same area as the ticks (Ardennes) did not reveal the presence of the virus (Cosson *et al*., unpublished data). In summary, there is still no clear evidence that the Eyach virus infects humans or animals, so this will require additional investigation.

Currently, new tick-borne viruses are often isolated from humans with febrile illness or death following a tick bite, as occurred for severe fever with thrombocytopenia syndrome virus in China [18], the Heartland virus [16], and the Bourbon virus in the USA [15]. But many other tick-borne viruses have been described without related human illness [17,33], leading to new challenges and questions in the field of tick-borne disease research: are those new viruses potential pathogens for humans, and even more importantly, could these new viruses be linked to human disease.

Acknowledgements

This work was partially funded by INRA, and by the EU grant FP7-261504 EDENext and is catalogued by the EDENext Steering Committee as EDENext421 (http://www.edenext.eu). We are grateful to Dr Sylvie Lecollinet, Dr Thomas Lilin and Dr Sabine Delannoy for their help and constant support. We thank the group *Tiques et Maladies à Tiques* (TMT) of *Réseau Ecologie des Interactions Durables* for stimulating discussion and support. This work was supported by the INRA MEM Metaprogramme (Patho-ID), the CoVetLab partner institutes, the ANSES, the Animal Health department of the INRA, the Laboratoire d'Excellence 'Integrative Biology of Emerging Infectious Diseases' (grant no. ANR-10-LABX-62-IBEID) and the COST Action TD1303 (EurNegVec).

Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.nmni.2016.02.012.

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